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ARTICLE

RanGTPase: A Candidate for Myc-Mediated Cancer Progression

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Background	Ras-related nuclear protein (Ran) is required for cancer cell survival in vitro and human cancer progression, but the molecular mechanisms are largely unknown.
Methods	We investigated the effect of the v-myc myelocytomatosis viral oncogene homolog (Myc) on Ran expression by Western blot, chromatin immunoprecipitation, and luciferase reporter assays and the effects of Myc and Ran expression in cancer cells by soft-agar, cell adhesion, and invasion assays. The correlation between Myc and Ran and the association with patient survival were investigated in 14 independent patient cohorts (n = 2430) and analyzed with Spearman's rank correlation and Kaplan–Meier plots coupled with Wilcoxon–Gehan tests, respectively. All statistical tests were two-sided.
Results	Myc binds to the upstream sequence of <i>Ran</i> and transactivates Ran promoter activity. Overexpression of Myc upregulates Ran expression, whereas knockdown of Myc downregulates Ran expression. Myc or Ran overexpression in breast cancer cells is associated with cancer progression and metastasis. Knockdown of Ran reverses the effect induced by Myc overexpression in breast cancer cells. In clinical data, a positive association between Myc and Ran expression was revealed in 288 breast cancer and 102 lung cancer specimens. Moreover, Ran expression levels differentiate better or poorer survival in Myc overexpressing breast ($\chi^2 = 24.1$; relative risk [RR] = 9.1, 95% confidence interval [CI] = 3.3 to 24.7, $P < .001$) and lung ($\chi^2 = 6.04$; RR = 2.8, 95% CI = 1.2 to 6.3; $P = .01$) cancer cohorts.
Conclusions	Our results suggest that Ran is required for and is a potential therapeutic target of Myc-driven cancer progression in both breast and lung cancers.
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Ras-related nuclear protein (Ran) GTPase is a small GTPase belonging to the Ras superfamily. Unlike other members, Ran lacks a cysteine residue at its C-terminus and hence does not undergo prenylation. Instead, it has an acidic C-terminal sequence—DEDDDL—that is essential for its cellular functions, including nucleocytoplasmic transportation, mitosis, and centrosome reduplication (1–3). The importance of Ran in tumorigenesis and as a cancer therapeutic target has been described in recent studies (4–6). Ran is overexpressed in cancer cell lines and tumor tissues at both the mRNA and protein levels compared with normal counterparts (4). Ran expression is required for mitosis of cancer cells but not normal cells (5,6). Cancer cells with K-Ras activating mutations are more dependent on Ran expression than their K-Ras wild-type counterparts (7,8). Ran expression is required for the survival of cancer cells with hyperactivation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways (8). Ran is also a poor prognostic marker in breast, lung, and ovarian cancers and renal cell carcinoma (8–10). In addition, we and others have shown that

Ran overexpression may play a role in the metastatic development of breast and lung cancers, highlighting a novel role of Ran in cancer progression (11,12).

The v-myc myelocytomatosis viral oncogene homolog (Myc) is an important oncogene that drives tumorigenesis by modulating various biological responses through transcriptional activation of its target genes (13). Recently, Myc expression has been shown to be associated with a “poor prognosis” signature, which is, in turn, associated with the development of distant metastases (14,15). Among other genes, Ran has been shown to be upregulated by Myc in a rat cDNA microarray study (16). Indeed, in silico prediction of transcription factor binding sites using Ran promoter sequence also revealed two potential Myc binding E-box elements (Supplementary Figure 1, available online). Given the important roles of Ran in cancer development and progression, it is important to study the transcriptional regulation of Ran expression, which may in turn explain its increased expression in cancer patients with poorer prognosis (8).

Methods and Materials

Cell Culture Conditions

Immortalized human breast epithelial cell line MCF10A and its Ras-transformed derivative MCF10AT (Karmanos Cancer Center, Detroit, MI; not authentically tested; cells were within 20 passages upon receipt from the donor) were maintained in Dulbecco's modified Eagle medium/F-12 containing 5% horse serum, 10 µg/mL insulin, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, and 0.5 µg/mL hydrocortisone. Viral Packaging cell lines 293T (American Type Culture Collection [ATCC] Manassas, VA) and Phoenix Ampho (gift from Nolan Laboratory, Stanford University, CA; not authentically tested; cells were within 20 passages upon receipt from the donor) were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Breast cancer cell lines MCF-7 (ATCC) and MDA MB 231 (ATCC) and lung cancer cell line A549 (ATCC) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Breast cancer cell line T47D (ATCC) was maintained in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum. MCF10AT and Phoenix Ampho cells were phenotypically verified. The cell lines from ATCC were authenticated by short tandem repeat profiling by ATCC and expanded upon receipt. Cell lines used in were within 30 passage upon receipt, and cells were split in a 3- to 5-day interval, and tested for mycoplasma contamination once a month.

5' Rapid Amplification of cDNA Ends (RACE) Assay

A 5' RACE assay was performed according to the manufacturer's instructions (Roche, Burgess Hill, UK) using the following three Ran promoter specific primers (RPSP): RPSP-1: 5'-CAGGTCATCATCCTCATCCGG-3'; RPSP-2: 5'-TGTTGCCACACAACAATG-3'; RPSP-3: 5'-CAAGGTGGCTACATACCTT-3'.

Plasmid

Ran cDNA was amplified by Phusion Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK) using total cDNA from MDA MB 231 cells and the forward primers RancdsF (5'-ATT GCG CTT CCG CCA TCT TT-3') and RancdsR (5'-GCT CCA GCT TCA TTC TCA CA-3'), and cloned into pBabe-puro retroviral over-expression vector. pBabe-Myc-zeo was a gift from R. Weinberg, and pRetrosuper shMyc was a gift from M. Eilers. pLKO.1-shRan1 (CCGGGCACAGTATGAGC-ACGACTTACTCGAGTAAGTCGTGCTCATACTGTGCTTTTGTG) and pLKO.1-shRan5 (CCGGCAGTTC-AACTTGATTTGGTTCTCGAGAACCAATACAA-GTTTGAAGTGTGTTTGTG) were obtained from Sigma-Aldrich (Dorset, UK). The Ran promoter (718 base pairs upstream of the transcription start site) was amplified using Phusion Hot Start High-Fidelity DNA Polymerase and cloned into pGL3 basic reporter construct (Promega, Southampton, UK).

Generation of Cell Lines

Transfection was performed using GeneJuice (Promega) according to the manufacturer's instruction. MCF10A pBabe-vector and MCF10A pBabe-Myc cells were generated by stable transfection by selecting cells in the appropriate amounts of antibiotics.

MCF10A-vector and MCF10A-Ran cells were generated by retroviral infection of pBabe-vector and pBabe-Ran plasmids, respectively. MDA MB 231-shScr (Scramble) and MDA MB 231-shcMyc cells were generated by retroviral infection of pRetrosuper-shScr and pRetrosuper-shcMyc, respectively. MDA MB 231-shScr and MDA MB 231-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan5, respectively, as previously described (8). The stable cells were used only when they were within 5 passages after establishment.

Western Blot

Western blotting was performed as previously described (8). Antibodies for Ran (from rabbits; Millipore, Watford, UK) were used at 1:1000 dilution; antibodies for Myc (from mice; Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:1000; and those for Actin (from mice; Sigma-Aldrich) were used at a dilution of 1:5000. The fold change in protein level was determined by densitometry.

Measurement of Ran Activity

Ran activity was measured using the Ran activation assay kit according to the manufacturer's instructions (Cell Biolabs, San Diego, CA). Briefly, GTP-bound Ran was pulled down by using RanBP1 PBD Agarose bead slurry, and the pulled-down complex was then dissociated by boiling in 2× reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer. The pull-down supernatant was then analyzed by Western blotting.

Soft Agar, Cell Adhesion, and Invasion Assays

These biological assays were performed as previously described (17). For soft agar assay, 5000 suspension cells in normal medium containing 0.35% (w/v) low-melting-point agarose were overlaid onto a solidified normal medium containing 0.7% (w/v) low-melting-point agarose. Cells were incubated at 37°C with 5% (v/v) carbon dioxide for 2 to 3 weeks. Colonies were visualized by staining with crystal violet and counted. For cell adhesion assay, 40 000 cells in suspension were seeded per well in a 96-well plate and were allowed to settle for 30 and 60 minutes. Suspended cells were removed by washing four times with phosphate-buffered saline, and adhered cells were then fixed with 70% (v/v) ethanol and stained with crystal violet. The excess crystal violet was washed away with deionized water, and the retained dye was extracted with 70% ethanol. The absorbance at 595 nm was measured in a microplate reader. For the invasion assay, 50 000 cells in serum- and phenol red-free medium were seeded into the upper layer of the transwell insert containing Matrigel (BD Biosciences, San Jose, CA). Invasion through Matrigel was allowed to take place for 24 hours with serum as the chemoattractant in the lower layer. Cells above the insert were removed, and those below were fixed and stained with crystal violet. Excess dye was washed away with water, and the retained dye was extracted. The absorbance at 595 nm was measured. All the biological assays were performed at 48 hours postinfection, where Ran knockdown had already been demonstrated, whereas Ran knockdown-induced cell death was not observed (Supplementary Figure 2, available online).

Luciferase Reporter Assay

Luciferase reporter assay was performed using the dual luciferase assay kit according to the manufacturer's instructions (Promega), as previously described [18]. Cells were seeded in a 12-well plate and cotransfected with the expression vectors and luciferase reporter plasmids after 24 hours. Protein was extracted after 36 hours, and the firefly luciferase activity was normalized to the internal control renilla luciferase activity.

Patients and Specimens

Patients and specimens were described previously (8). A total of 320 formalin-fixed, paraffin-embedded breast carcinoma specimens were taken from an archive that was collected between 1976 and 1986 at the Breast Unit, Royal Liverpool Hospital. The mean age of the patients was 57 years (range = 29–92 years). No sign of metastasis was seen in these patients before specimen procurement. Treatment was either mastectomy or radical mastectomy with no prior endocrine or other systemic therapy. Follow-up data were collected between 14 and 20 years after treatment had been administered. All patient data were made confidential by using an anonymized system by the Merseyside Cancer Registry. A total of 113 formalin-fixed, paraffin-embedded early-stage, non-small cell lung cancer specimens obtained from Dublin St. James' Hospital and resected by either lobectomy or pneumonectomy were incorporated into a tissue microarray for immunohistochemical staining. Among these patients, 109 had not received any form of adjuvant chemotherapy before the procurement of the specimens. Anonymized follow-up information, including patient survival, histology, and tumor node metastasis classification of malignant tumors staging were provided by the Northern Ireland Cancer Registry. Informed consent was obtained from patients. The Liverpool breast cancer patient cohort was approved by the Liverpool Adult Research Ethics Committee (07/H1005/93), whereas the Dublin lung cancer patient cohort was approved by Saint James's Hospital and Adelaide and Meath Hospital, Dublin (TCD041018/8804).

Immunohistochemistry

Immunohistochemical staining was performed as previously described (8) according to the manufacturer's instructions (DAKO Envision+ kit; DAKO, Ely, UK). Ran antibody was used at a dilution of 1:100, whereas Myc antibody was used at a dilution of 1:250.

Evaluation of Immunohistochemical Staining Results

Evaluation was performed as previously described (8). For breast cancer specimens (from Liverpool), the whole section was used for staining, and the percentage of stained carcinoma (both nuclei and cytoplasm) cells was recorded from at least 10 fields of duplicate histological sections at 200× magnification by two independent observers and the average result obtained. For analysis, the tumors were divided into two groups, classified as immunohistochemically negative if less than 1% of carcinoma cells were stained and immunohistochemically positive if greater than 1% of carcinoma cells were stained. For lung cancer specimens (from Dublin), which were incorporated into the tissue microarray, the expression of nuclear and cytoplasmic Ran and nuclear Myc was scored from 0 to 5 based on the extent and intensity of the staining

by two independent observers (H.-F. Yuen and K. O'Byrne.). The score was agreed by the two observers. All three cores of each specimen in the tissue microarray were scored, and the mean score for each specimen was obtained. The mean score was agreed upon again by the two observers. The mean score was further classified into high- and low-level expression with a cutoff point at 2.5. For overall Ran expression, tumors with high levels of both nuclear and cytoplasmic Ran staining were considered as high, tumors with high level of nuclear and low level of cytoplasmic Ran, or vice versa, were considered as intermediate, and tumors with low levels of both nuclear and cytoplasmic Ran were considered as low.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, NY). Differences between groups in *in vitro* experiments were tested by Student *t* test (two groups) and analysis of variance (ANOVA) test with post hoc Games–Howell (comparing two groups when more than three groups were tested in the same experiment). Differences in expression levels between groups/samples in the human specimens were analyzed by χ^2 , Fisher exact test, or Mann–Whitney *U* tests, where applicable. The association between the expression level and patient survival was recorded by Kaplan–Meier plots and compared by Wilcoxon–Gehan tests. A *P* value of less than .05 was considered statistically significant. All statistical tests were two-sided.

Analysis of Breast and Lung Cancer Microarray Data

Seven breast cancer datasets [GSE1456 (19), GSE2034 (20), GSE3143 (21), GSE4922 (22), GSE7390 (23), GSE11121 (24), GSE12276 (25)], each consisting of more than 150 patients, were included in the analysis. Five lung cancer datasets [GSE3141 (21), GSE4573 (26), GSE8894 (27), GSE13213 (28), GSE14814 (29)], each consisting of more than 90 patients, were included in the analysis. The datasets were preprocessed as previously described using R and Bioconductor for normalization (8). The combined breast cancer dataset consisted of 1454 patients, whereas the combined lung cancer dataset consisted of 586 patients. The patients were stratified equally into four groups based on the expression levels of Ran and Myc (each group contains 25% of the patients) for further statistical analysis. The methodology for the bioinformatics steps is provided as Sweave documentation in the [Supplementary Methods](#) (available online).

Results

Effect of Myc on Ran Transcription

Ran has been shown to be upregulated by Myc in a rat cDNA microarray study on identification of Myc downstream targets (16), whereas our *in silico* analysis of the Ran upstream sequence revealed two Myc binding E-box elements ([Supplementary Figure 1](#), available online). These results led us to investigate whether Myc is a transcriptional activator of Ran. As shown in [Figure 1A](#), overexpression of Myc by 2.1-fold resulted in upregulation of endogenous Ran protein in the breast MCF10AT cell line by 2.3-fold as determined by Western blot analysis. The increase in the active form of Ran (RanGTP) was also detected upon Myc overexpression

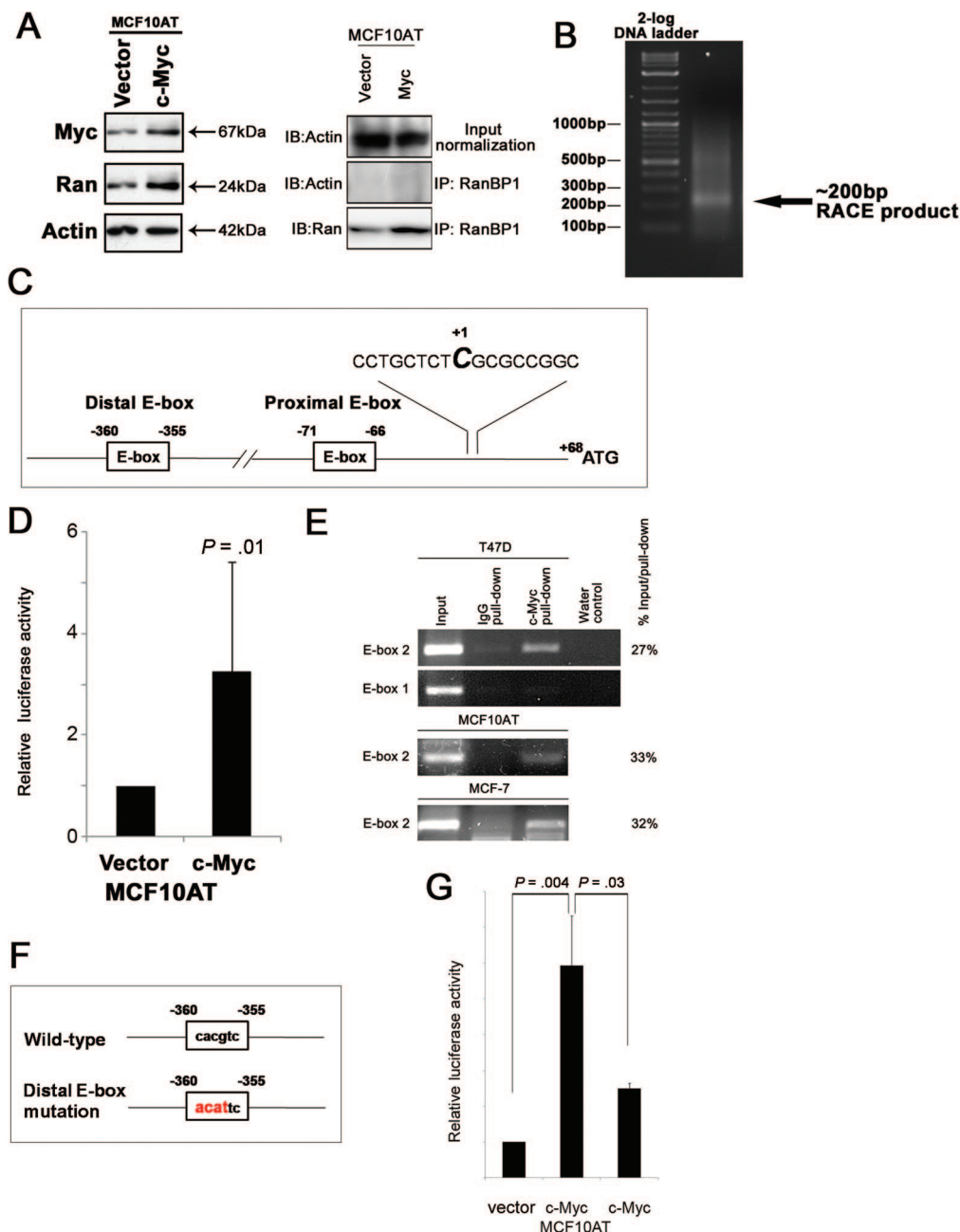


Figure 1. Transcriptional regulation of Ras-related nuclear protein (Ran) by v-myc myelocytomatosis viral oncogene homolog (Myc). **A** **Left panel**, Western blot of Myc-transfected cells. Stable Myc over-expressing MCF10AT cells had a higher level of Myc (2.1-fold) and a higher endogenous protein level of Ran (2.3-fold) compared with the vector control MCF10AT cells. **Right panel**, Immunoprecipitation (IP) was performed using anti-Ran binding protein 1 (RanBP1) antibody, which binds to Ran (binds only to RanGTP), in both MCF10AT-vector and MCF10AT-Myc cells. The resultant pull-down eluents were then analyzed by immunoblotting (IB) for Ran. Actin was used as a loading control for inputs and negative control for the pull-down assay for the two cell lines. **B** Gel electrophoresis of the polymerase chain reaction (PCR) product from 5' Rapid Amplification of cDNA Ends (RACE) assay showing a specific PCR product of approximately 200 base pairs (bp). **C** A schematic diagram of Ran promoter. The cysteine residue at +1 position is the transcriptional start site of the *Ran* gene identified by sequencing of the PCR product obtained in (B). **D** Ran promoter activity. Transfection of expression vector for Myc and the Ran promoter reporter construct in MCF10AT cells resulted in a statistically significant increase in the luciferase reporter assay for the Ran promoter activity compared with transfection of control vector and Ran promoter reporter

construct (mean increase = 3.2-fold, 95% confidence interval [CI] = 1.1 to 5.4; Student *t* test, $P = .01$). The histogram is showing mean and 95% confidence interval for the promoter activity. **E** Chromatin immunoprecipitation (ChIP) assay for the Ran promoter region. Myc binds to the distal E-box element in human Ran promoter in vivo. Positive control template was input genomic DNA, and negative control template was water. PCR of immunoglobulin G (IgG) pull-down ChIP products was used as a control to the Myc pull-down ChIP products for each cell line and are shown side-by-side. **F** A schematic diagram for distal E-box mutagenesis of Ran promoter. Mutations were introduced into the pGL3-RanPro reporter construct. **G** Activities of wild-type (WTpro) and mutant (Mutpro) Ran promoter. Myc overexpression resulted in a statistically significant increase in relative luciferase activity driven by the Ran promoter in MCF10AT cells (mean increase = 5.9-fold, 95% CI = 4.54 to 7.31; analysis of variance [ANOVA] post hoc Games-Howell test, $P = .004$). This Myc-mediated increase in Ran promoter activity was significantly reduced to 42% of the WT counterpart (95% CI = 33% to 53%; ANOVA post hoc Games-Howell test, $P = .03$) when the sequence of the distal E-box element of the pGL3-Ranpro construct was mutated. The histogram is showing mean and 95% confidence interval for the promoter activity. All statistical tests were two-sided.

in MCF10AT cells by the Ran activation assay (Figure 1A, right panel; twofold increase). The transcriptional start site of Ran was identified by using the 5'RACE assay, yielding a specific DNA band of about 200 base pairs as the 5'RACE polymerase chain reaction product (PCR) (Figure 1B). By sequencing this PCR product, a cytosine residue at 68 base pairs upstream of the translational initiation site was identified as the transcriptional start site of the *Ran* gene in MCF10AT cells (Figure 1C; Supplementary Figure 3, available online). Based on the in silico analysis of the upstream sequence from the *Ran* gene and the 5'RACE assay, we cloned the putative promoter of Ran, Ranpro, into the luciferase reporter construct, pGL3, to produce pGL3-Ranpro by PCR amplification using MCF10AT genomic DNA. Using the dual luciferase assay, Ran promoter activity was increased by 3.2-fold (95% confidence interval [CI] = 1.1 to 5.4; $P = .01$, Student *t* test) (Figure 1D) when Myc was overexpressed in MCF10AT cells.

In MCF10AT, T47D, and MCF-7 breast cancer cell lines, the Ran promoter region containing the distal E-box was enriched in Myc chromatin immunoprecipitation (ChIP) products (Figure 1E). ChIP assay using Myc antibody resulted in 27% to 33% pull-down efficiency of the Ran promoter region containing the distal E-box element in all three breast cancer cell lines tested (Figure 1E). In contrast, the Ran promoter region containing the proximal E-box element was not enriched in the same Myc ChIP products (Figure 1E). Our results suggest that Myc binds to this distal E-box element, but not to the proximal E-box element, in vivo in these three cell lines. The activity of the Ran promoter was statistically significantly increased when Myc was overexpressed (mean = 5.9-fold, 95% CI = 4.54 to 7.31; $P = .004$, ANOVA post hoc Games–Howell test), and this increase was statistically significantly reduced to 42% of its wild-type counterpart (95% CI = 33% to 53%; $P = .03$, ANOVA post hoc Games–Howell test) when the distal E-box element was mutated compared with the wild-type Ran promoter (Figure 1, F and G). Together, our results suggest that Myc binds to the distal E-box element in the Ran promoter region to activate expression of Ran, which results in an increase in activity of Ran (RanGTP).

Cell Biological Properties Associated With Modulation of Myc and Ran Expression

When Myc was overexpressed in an immortalized but otherwise normal breast epithelial cell line, MCF10A, endogenous Ran expression was increased (Figure 2A). Overexpression of Myc resulted in a statistically significant increase in growth of MCF10A cells in soft agar (2.5-fold increase, 95% CI = 1.8 to 3.2; $P = .008$, Student *t* test) (Figure 2B). In addition, knockdown of Myc in an invasive breast cancer cell line MDA MB 231 cells resulted in downregulation of Ran (Figure 2C) and a concomitant statistically significant decrease in the number of colonies formed in soft agar to 40% of control (95% CI = 33% to 48%; $P = .007$, Student *t* test) (Figure 2D), in cell invasion through Matrigel-coated transwell (mean = 65% of the control cells, 95% CI = 62% to 68%; $P = .008$, Student *t* test) (Figure 2E), and in cell adhesion to fibronectin-coated plate (mean = 58% of control cells, 95% CI = 47% to 70%; $P = .04$, Student *t* test) (Figure 2F), which are cellular properties associated with the metastatic state in vitro.

Overexpression of Ran also resulted in a statistically significant increased number of colonies formed by MCF10A cells in soft agar

(mean = 7.1-fold, 95% CI = 3.7 to 10.5; $P = .01$) (Figure 3, A and B). We have shown previously that silencing of Ran results in appreciable apoptosis, which greatly interferes with the interpretation of data for investigation of cellular properties associated with metastasis in vitro (8). To avoid induction of severe apoptosis while maintaining a significant level of Ran knockdown, the viral titer was reduced and a less potent small hairpin (sh) RNA targeting Ran, shRan5 was used. This protocol resulted in a significant downregulation of Ran in MDA MB 231 breast cancer cells without appreciably inducing apoptosis, even after 96 hours of viral infection (Supplementary Figure 2, available online). Knockdown of Ran in invasive MDA MB 231 cells led to a statistically significant decrease in colony formation of cancer cells in soft agar to 19% of the control cells (95% CI = 17% to 21%; $P < .001$, Student *t* test) (Figure 3, C and D), in cell invasion through Matrigel-coated transwell membranes (38% of the control cells, 95% CI = 15% to 64%; $P = .03$, Student *t* test) (Figure 3E) and in cell adhesion to fibronectin-coated 96-well plates (at 30 minutes, 70% of the control cells, 95% CI = 62% to 78%; at 60 minutes, 69%, 95% CI = 61% to 77%; Student *t*-test, $P = .03$) (Figure 3F). These results suggest that Myc and Ran can separately stimulate similar cellular properties that are associated with cancer progression and metastasis. Overexpression of Ran in MCF10AT or knockdown of Ran in MDA MB231 cells did not change the expression of Myc (Supplementary Figure 4, available online), suggesting that the regulatory link between Myc and Ran is unidirectional.

Effect of Ran in Myc-Mediated Cancer Cell Tumorigenesis

Overexpression of Myc resulted in upregulation of endogenous Ran in MCF10A immortalized breast epithelial cells and this effect was reversed, as shown by Western blot analysis, when the cells were infected with the above shRNA targeting Ran, shRan5 (Figure 4A). Moreover, when Myc was overexpressed in MCF10A cells, their ability to form colonies in soft agar was statistically significantly reversed by knocking down expression of Ran (Myc overexpression: mean = 3.6-fold, 95% CI = 3.0 to 4.3, $P = .002$; Myc overexpression with Ran knockdown: mean = 0.75, 95% CI = 0.41 to 1.09, $P = .13$; ANOVA post hoc Games–Howell test) (Figure 4B). We then further investigated the importance of Ran in two more breast cancer cell lines, MCF-7 and T47D, in which their tumorigenicity has been shown to be positively associated with Myc expression levels (30–33). Knockdown of Ran in these two breast cancer cell lines (Figure 4, C and E) resulted in a statistically significant decrease in their ability to form colonies in the soft agar assay (MCF-7: mean = 9% of control cells, 95% CI = 6% to 12%, $P = 0.003$; T47D: mean = 11% of control cells, 95% CI = 5% to 18%, $P = 0.006$, Student *t* test) (Figure 4, D and F). Together, these results suggest that Ran expression is important for these breast cancer cell lines to maintain their tumorigenicity in vitro.

Association Between Myc and Ran Expression in Human Breast Cancers

Immunohistochemical staining for nuclear Myc protein was highly statistically significantly associated with staining for nuclear Ran (Fisher exact test, $P < .001$), with staining for cytoplasmic Ran ($P < .001$), and with overall staining for Ran ($P < .001$) (Figure 5, A–H).

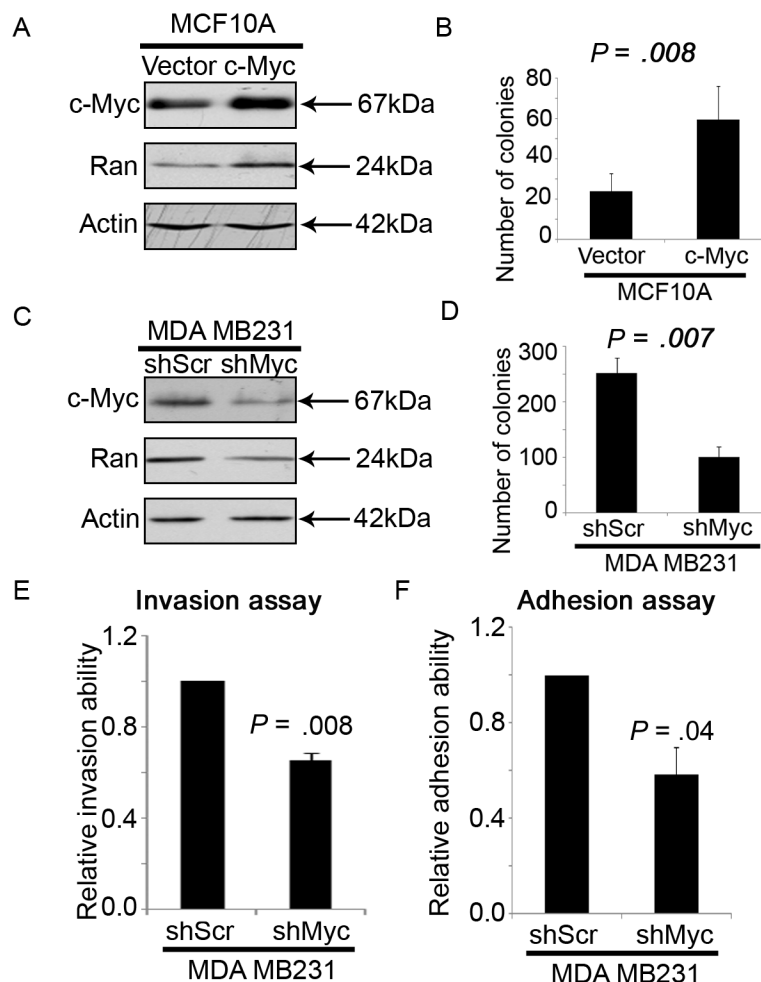


Figure 2. Effect of v-myc myelocytomatosis viral oncogene homolog (Myc) on in vitro biological properties associated with cancer progression. **A)** Western blots for MCF10A-vector control and MCF10A-Myc cells. MCF10A-vector control and MCF10A-Myc cells were generated by transfection of pBabe-zeo and pBabe-zeo-Myc, respectively, followed by selection in zeocin. Stable overexpression of Myc in MCF10A cells resulted in upregulation of both Myc (1.7-fold) and the endogenous Ras-related nuclear protein (Ran) (2.1-fold). **B)** Soft agar assay for MCF10A-vector and MCF10A-Myc cells. Histogram showing mean and 95% confidence interval (CI) for the number of colonies formed in soft agar from three independent experiments. The number of colonies was statistically significantly greater for MCF10A-Myc cells than for MCF10A-vector control cells (2.5-fold, 95% CI = 1.8 to 3.2; Student *t* test, $P = .008$). **C)** Western blots for MDA MB 231-shScr and MDA MB 231-shMyc cells. MDA MB 231-shScr and MDA MB 231-shMyc cells were generated by retroviral infection of pRetrosuper-shScramble RNA and pRetrosuper-shMyc RNA, respectively, followed by selection in puromycin. Knockdown of Myc by retroviral infection of Myc shRNA resulted in downregulation of both the endogenous Myc (61% reduction) and the endogenous Ran (30% reduction) protein levels. **D-F)** Assays for in vitro

biological properties associated with cancer progression and metastasis of MDA MB 231-shScr and MDA MB 231-shMyc cells. **D)** Histogram showing mean and 95% confidence interval for the number of colonies formed in soft agar from three independent experiments. MDA MB 231-shMyc cells had a statistically significantly decreased number of colonies formed in soft agar assay relative to MDA MB 231-shScr cells (40% of control, 95% CI = 33% to 48%; Student *t* test, $P = .007$). **E)** The same number of MDA MB 231 cells was seeded in the upper chamber of an invasion chamber. They were allowed to migrate through the Matrigel-coated membrane overnight using fetal bovine serum as chemoattractant. MDA MB 231-shMyc cells had a statistically significant decrease in ability to invade through Matrigel compared with MDA MB 231-shScr cells (65% of the control cells, 95% CI = 62% to 68%; Student *t* test, $P = .008$). **F)** Cells adhering to the fibronectin-coated wells were fixed with 70% ethanol, washed, and then stained with crystal violet. The retained crystal violet was extracted and the absorbance was measured. MDA MB 231-shMyc cells had a statistically significant decrease in ability to adhere to fibronectin compared with MDA MB 231-shScr cells (58% of control cells, 95% CI = 47% to 70%; Student *t* test, $P = .04$). All statistical tests were two-sided.

Moreover, when the mRNA levels of Ran and Myc from seven independent breast cancer datasets were analyzed, statistically significant positive correlations between the mRNA levels of these two genes were observed in all seven individual datasets (Spearman rank test, $P < .05$) (Figure 5, I–O). When the mRNA levels were stratified into four groups evenly and the seven datasets were combined ($n = 1454$), the positive association between Ran and Myc mRNA levels was highly statistically significant (χ^2 test, $P < .001$) (Figure 5P). These results strongly demonstrate the

positive association between nuclear Myc and Ran expression in patient specimens.

Association of Ran Expression and Patient Survival in Myc-Overexpressing Breast Cancers

A statistically significant association between positive nuclear staining for Myc and a shorter survival time of the patients was observed (Wilcoxon–Gehan test, $P < .001$) (Figure 6A). The median survival times for patients with positive and negative staining tumors for

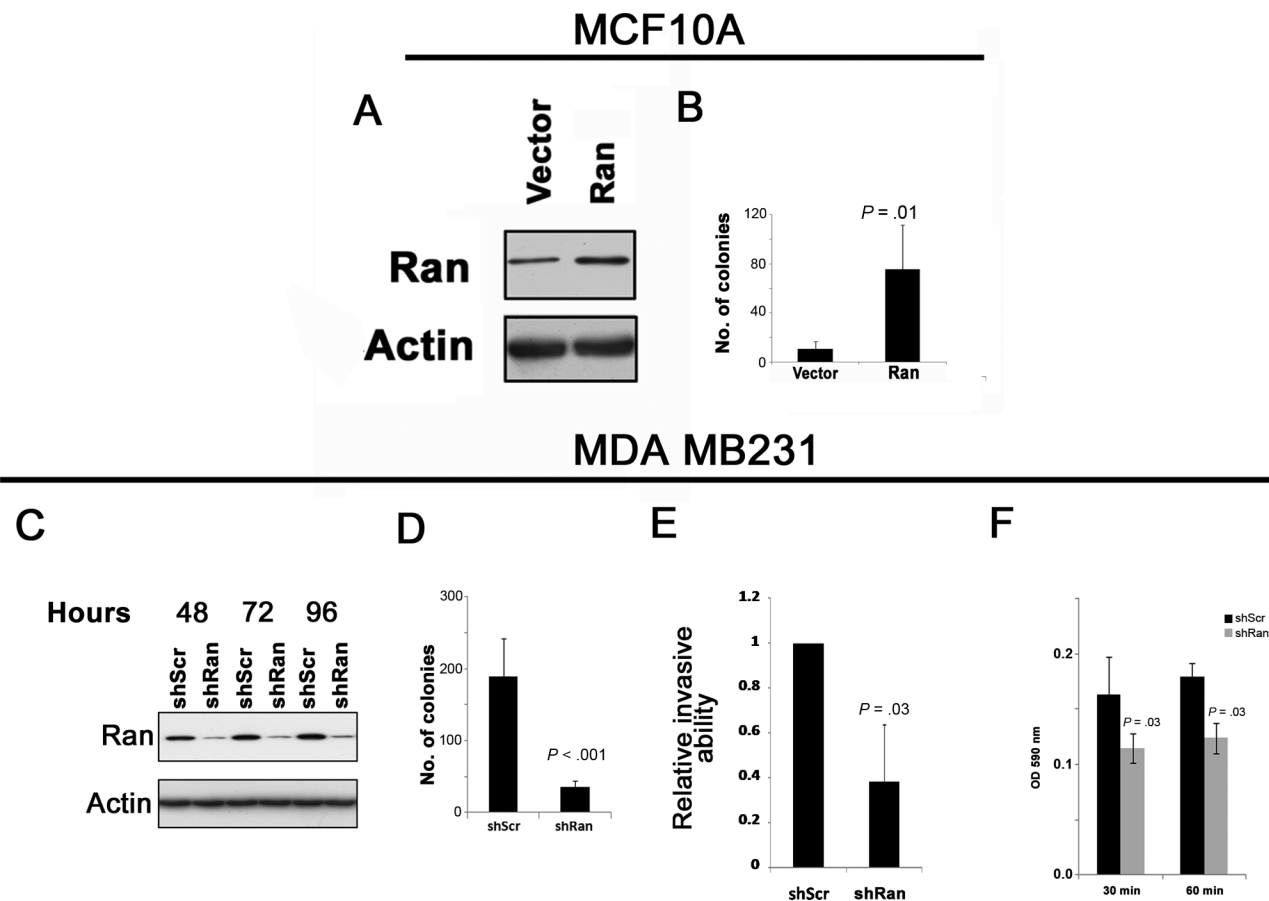


Figure 3. Effect of Ras-related nuclear protein (Ran) on in vitro biological properties associated with cancer progression and metastasis. **A**) Western blots for MCF10A-vector and MCF10A-Ran cells. MCF10A-vector control and MCF10A-Ran cells were generated by retroviral infection of pBabe-hygro and pBabe-hygro-Ran, respectively, followed by selection in hygromycin. A higher protein level of Ran (2.1-fold) was detected in MCF10A-Ran cells compared with MCF10A-vector control cells. **B**) Soft agar assay for MCF10A-vector and MCF10A-Ran cells. Histogram showing mean and 95% confidence interval (CI) for the number of colonies formed in soft agar from three independent experiments. MCF10A-Ran cells had a statistically significant 7.1-fold (95% CI = 3.7 to 10.5-fold; Student *t* test, *P* = .01) increase in ability to form colonies in soft agar assay compared with MCF10A-vector control cells. **C**) Western blots for MDA MB 231–short hairpin Scramble (shScr) and MDA MB 231–short hairpin RNA against Ran (shRan) cells. MDA MB 231-shScr and MDA MB 231-shRan cells were generated by lentiviral infection of pLKO.1-shScramble RNA and pLKO.1-shRan RNA, respectively, followed by

selection in puromycin. MDA MB 231-shRan cells had a lower level of Ran protein at various times after infection (90%, 87%, and 83% reduction at 48, 72, and 96 hours, respectively). **D–F**) Assays for biological properties associated with cancer progression and metastasis of MDA MB 231-shScr and MDA MB 231-shRan cells. **D**) Histogram showing mean and 95% confidence interval for the number of colonies formed in soft agar from three independent experiments. MDA MB 231-shRan cells formed statistically significantly fewer colonies in soft-agar compared with MDA MB 231-shScr cells (19% of the control cells, 95% CI = 17% to 21%; Student *t* test, *P* < .001). **E**) MDA MB 231-shRan cells had a statistically significant reduced ability of invading through Matrigel compared with MDA MB 231-shScr cells (38% of the control cells, 95% CI = 13% to 64%; Student *t* test, *P* = .03). **F**) MDA MB 231-shRan cells had a statistically significantly decreased ability to adhere to fibronectin compared with MDA MB 231-shScr cells (at 30 minutes: 70% of the control cells, 95% CI = 62% to 78%; at 60 min: 69%, 95% CI = 61% to 77%; Student *t* test, *P* = .03). All statistical tests were two-sided.

nuclear Myc were 70.13 and more than 228 months, respectively. In contrast, cytoplasmic staining for Myc was not statistically significantly associated with patient survival time (Wilcoxon–Gehan test, *P* = .34) (Figure 6B). Using Cox univariable regression analysis, positive staining for nuclear Myc was statistically significantly associated with an increased risk of patient death of 10.6-fold (95% CI = 5.2 to 21.8; *P* < .001). These results suggest that an increased expression of Myc may promote metastatic progression of breast cancer, possibly through its nuclear rather than its cytoplasmic activity.

In our cohort of breast cancer patients with positive staining for nuclear Myc, the association between positive staining for nuclear Ran and survival was highly statistically significant ($\chi^2 = 24.1$; relative risk [RR] = 9.10, 95% CI = 3.3 to 24.7; *P* < .001) (Figure 6C). In this group of patients, the median survival time for those with

positive staining for nuclear Ran was 56 months, whereas it was 216 months for those with negative staining. In contrast, much less difference in median survival time between positive and negative nuclear Ran staining was observed in those patients with negative staining for nuclear Myc. The median survival time for those with positive staining for nuclear Ran was 204 months, and it was more than 228 months for those with negative staining (Figure 6D). Likewise, in patients with positive staining for nuclear Myc, the association between positive staining for cytoplasmic Ran and survival was statistically significant ($\chi^2 = 9.664$; RR = 2.08, 95% CI = 1.41 to 3.08; *P* = .002) (Figure 6D). In this group of patients, the median survival time for those with positive staining for cytoplasmic Ran was 56 months, whereas it was 190 months for those with negative staining. In contrast, much less difference in median

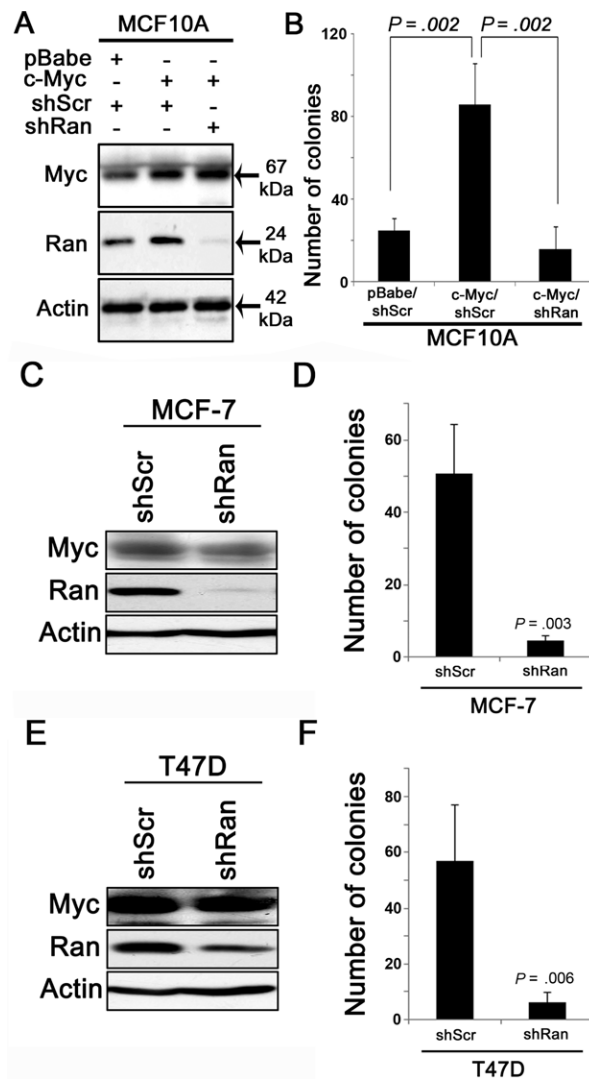


Figure 4. Effect of Ras-related nuclear protein (Ran) on v-myc myelocytoma virus oncogene homolog (Myc)-driven in vitro biological properties associated with cancer progression. **A)** Western blots for MCF10A-vector control–short hairpin Scramble (shScr), MCF10A-Myc-shScr and MCF10A-Myc–short hairpin RNA against Ran (shRan) cells. MCF10A-vector control-shScr, MCF10A-Myc-shScr, and MCF10A-Myc-shRan cells were generated by lentiviral infection of pLKO.1-shScr, pLKO.1-shScr, and pLKO.1-shRan, respectively, followed by selection in puromycin. MCF10A-Myc-shScr cells had a higher level of endogenous Ran protein (1.6-fold) compared with MCF10A-vector control-shScr cells. This increase in Ran protein level was abolished in MCF10A-Myc-shRan cells upon infection of pLKO.1-shRan construct (87% reduction). **B)** Histogram showing mean and 95% confidence interval (CI) from three independent experiments for the results from the soft agar assay for MCF10A-vector control-shScr, MCF10A-Myc-shScr, and MCF10A-Myc-shRan cells. MCF10A-Myc-shScr cells had a statistically significant 3.6-fold (95% CI = 3.0 to 4.3; analysis of variance [ANOVA] post hoc Games–Howell test, $P = .002$) increased ability to form colonies in soft agar assay. This increase in soft agar colony formation was reduced in MCF10A-Myc-shRan cells, which resulted in no statistically

significant difference to MCF10A-vector control-shScr cells (mean = 75% of control, 95% CI = 41% to 109%; ANOVA post hoc Games–Howell test, $P = .13$). **C)** Western blots for MCF-7-shScr and MCF-7-shRan cells. MCF-7-shScr and MCF-7-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. Myc, Ran, and Actin expression levels for these two cell lines were assessed by Western blot. **D)** Histogram showing mean and 95% confidence interval from three independent experiments for the results from the soft agar assay for MCF-7-shScr and MCF-7-shRan cells (mean = 9% of control cells, 95% CI = 6% to 12%; Student t test, $P = .003$). **E)** Western blots for T47D-shScr and T47D-shRan cells. T47D-shScr and T47D-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. Myc, Ran, and Actin expression levels for these two cell lines were assessed by Western blot. **F)** Histogram showing mean and 95% confidence interval from three independent experiments for the results from the soft agar assay for T47D-shScr and T47D-shRan cells (mean = 11% of control cells, 95% CI = 5% to 18%; Student t test, $P = .006$). All statistical tests were two-sided.

survival time between positive and negative cytoplasmic staining for Ran was observed in those patients with negative staining for nuclear Myc. The median survival time for those with positive staining for cytoplasmic Ran was 204 months, and it was more than 228 months for those with negative staining (Figure 6D). In the combined breast cancer datasets, a high level of Ran mRNA was also statistically significantly associated with a shorter survival time (7 independent datasets, $n = 1454$; $\chi^2 = 14.26$; RR = 1.35,

95% CI = 1.16 to 1.58; $P < .001$) (Supplementary Figure 5A, available online). Moreover, a high level of Ran mRNA was statistically significantly associated with a higher relative risk ($n = 728$; $\chi^2 = 8.30$; RR = 1.41, 95% CI = 1.12 to 1.78; $P = .004$) (Figure 6G) in patients expressing a high level of Myc mRNA and was less potent in those patients expressing a low level of Myc mRNA ($n = 726$; $\chi^2 = 4.39$; RR = 1.28, 95% CI = 1.02 to 1.61; $P = .04$) (Figure 6H).

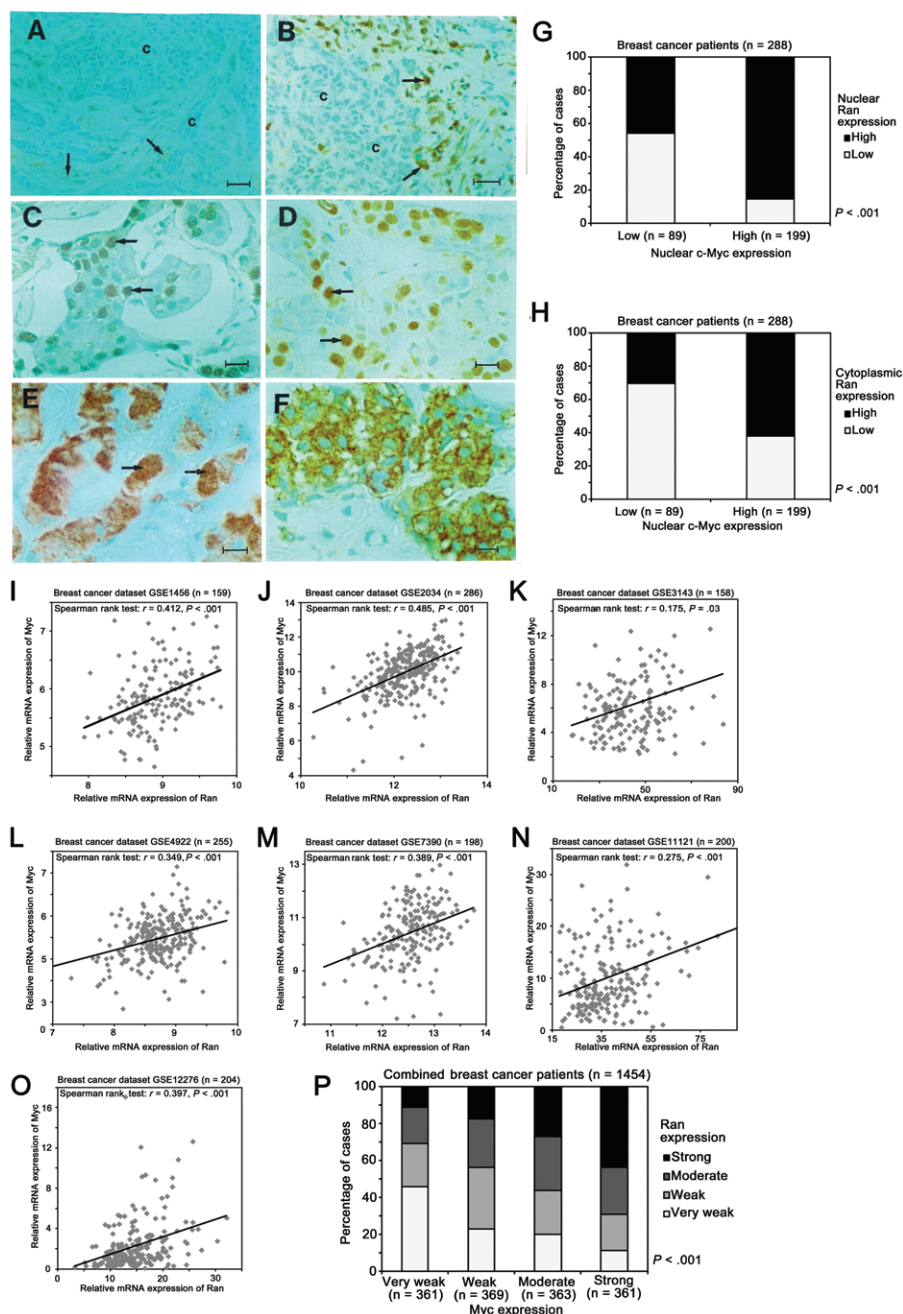


Figure 5. Association of protein and mRNA levels of v-myc myelocytomatosis viral oncogene homolog (Myc) and Ras-related nuclear protein (Ran) in breast cancer specimens. **A–F** Representative images of immunohistochemical staining of breast cancers for Myc (**A, C, E**) and Ran (**B, D, F**). Two separate histological sections from each of three tumors are shown: tumor 1 (**A, B**), tumor 2 (**C, D**), tumor 3 (**E, F**). Sections were immunohistochemically stained for Myc (**A, C, E**) and for Ran (**B, D, F**). In tumor 1, carcinoma cells (c) were unstained for Myc (**A**) and for Ran (**B**), whereas reactive stromal cells (arrows) were well stained with either antibody. In tumor 2, nuclei of carcinoma cells were well stained for Myc (**C**) (arrows) and for Ran (**D**) (arrows); there was little or no staining of carcinoma cell cytoplasm with either antibody. In tumor 3, the cytoplasm and some nuclei (**E**) (arrows) of the carcinoma cells were stained for Myc, whereas only the cytoplasm (**F**) was stained for Ran. Magnification 390 \times (**A–D**), 960 \times (**E, F**). Scale bars, 25 μ m (**A–D**); 10 μ m (**E, F**). **G** and **H** Comparison of immunohistochemical staining in all the 288 different tumors for staining for nuclear Myc and for nuclear Ran (**G**) and staining for nuclear Myc and for cytoplasmic Ran (**H**). Fisher exact test was used. **I–P** Comparison of relative levels of Myc and Ran mRNA in breast cancer datasets available in the

Gene Expression Omnibus (GEO) database. Relative mRNA levels of Myc and Ran was extracted from the GEO database and normalized by using R and Bioconductor as previous described (8). Datasets with patient number greater than or equal to 150 were included, and the correlations between relative mRNA levels of Myc and Ran in each dataset were compared by nonparametric bivariable correlation analysis (Spearman rank test) using SPSS. **I–O** In all the datasets tested in this study—GSE1456 ($n = 159$) (**I**), GSE2034 ($n = 286$) (**J**), GSE3143 ($n = 158$) (**K**), GSE4922 ($n = 255$) (**L**), GSE7390 ($n = 198$) (**M**), GSE1121 ($n = 200$) (**N**), and GSE12276 ($n = 204$) (**O**)—the relative mRNA levels of Myc and Ran were significantly positively correlated (Spearman rank test; $P < .05$). **P** Histogram showing percentage of cases with different levels of Ran mRNA in patients with different levels of Myc mRNA. The seven datasets were combined into one by stratifying the mRNA levels of Myc and Ran into four groups equally using the three quartiles as cut-off points. In the combined breast cancer dataset ($n = 1454$), the association between mRNA levels of Myc and Ran was tested by Spearman rank test. Statistically significantly more patients with a higher level of Myc mRNA also had a higher level of Ran mRNA (χ^2 test; $P < .001$). All statistical tests were two-sided.

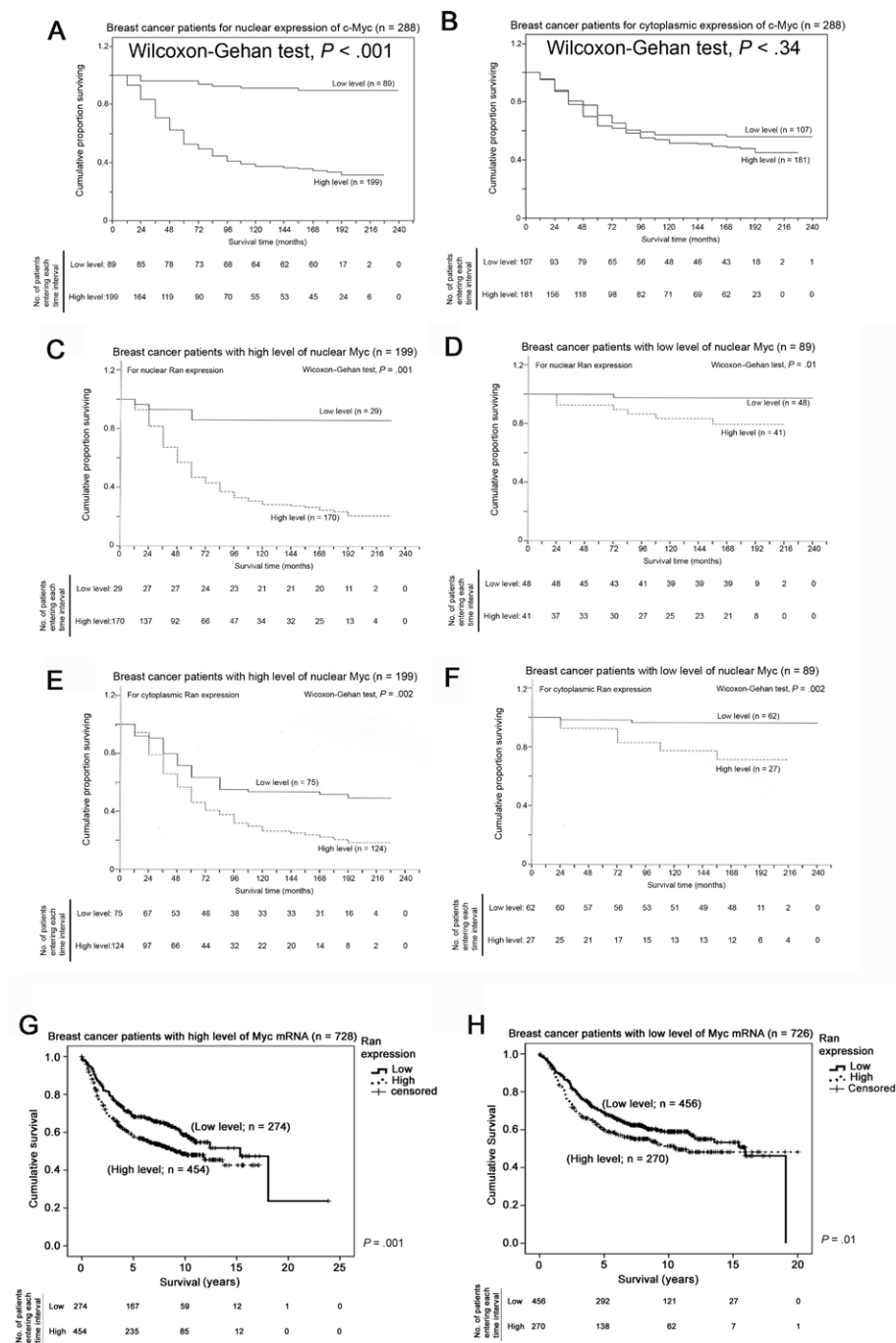


Figure 6. Association of expression of v-myc myelocytomatosis viral oncogene homolog (Myc) and Ras-related nuclear protein (Ran) alone and in combination with patient survival in breast cancer. **A–F** Kaplan–Meier plots of the cumulative proportion of patients surviving with time in months of the Liverpool breast cancer patient cohort (n = 288) are shown for tumors with nuclear Myc alone (**A**), tumors stained for cytoplasmic Myc alone (**B**), nuclear Myc-positive tumors with either positive or negative staining for nuclear Ran (**C**), nuclear Myc-negative tumors with either positive or negative staining for nuclear Ran (**D**),

nuclear Myc-positive tumors stained for either positive or negative staining for cytoplasmic Ran (**E**), and nuclear Myc-negative tumors stained for either positive or negative staining for cytoplasmic Ran (**F**). **G, H** Kaplan–Meier plots of cumulative proportion of patients surviving with time in years of the combined breast cancer dataset (n = 1454) from the Gene Expression Omnibus database for mRNA level of Ran in patients with a high level (**G**) and a low level (**H**) of Myc mRNA expression. Wilcoxon–Gehan test was used. All statistical tests were two-sided.

Correlation Between Myc and Ran Expression in Human Lung Cancers

In A549 lung cancer cells, overexpression of Myc also transactivates the Ran promoter activity in vitro by luciferase reporter assay (Supplementary Figure 6A, available online). Myc expression in

A549 cells was shown to be associated with an increase proliferation and tumorigenesis (34). We also found that knockdown of Ran in this cell line resulted in a statistically significant reduction in the ability of the cells to form colonies in soft agar (9% of the control cells, 95% CI = 6% to 12%; $P = .002$) (Supplementary Figure 6, B

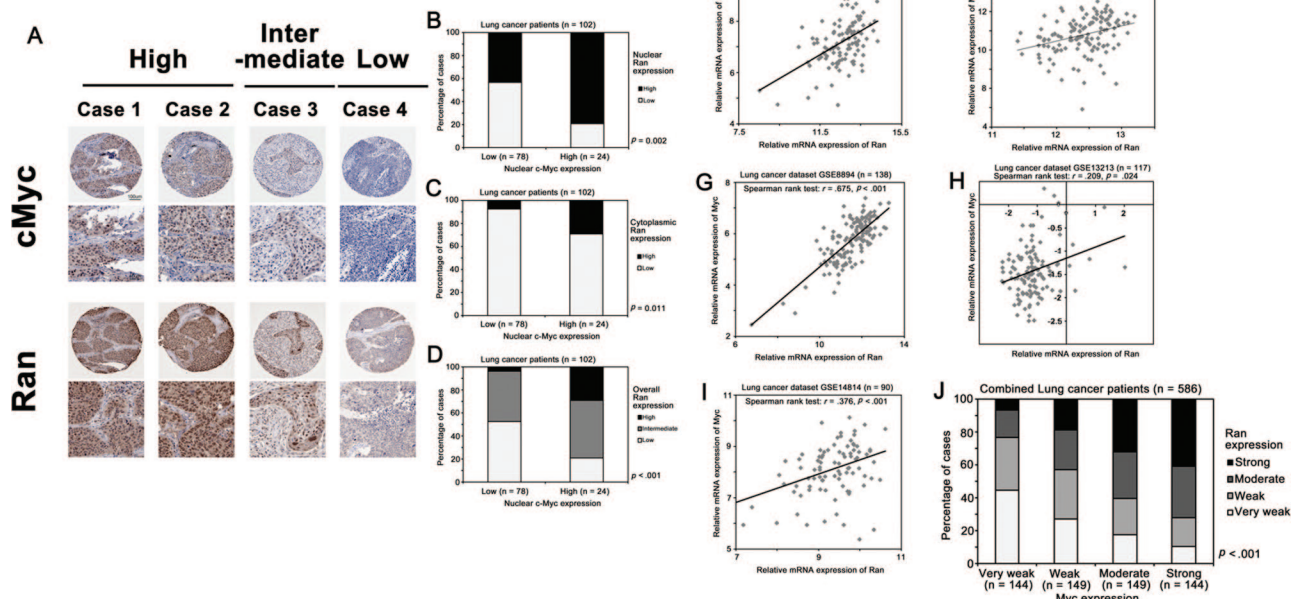


Figure 7. Association of expression of v-myc myelocytomatosis viral oncogene homolog (Myc) and Ras-related nuclear protein (Ran) in lung cancer patients. **A**) Representative images of immunohistochemical staining for Myc and Ran in lung cancer specimens. **Top panel:** scale bar = 100 μ m; **bottom panel:** scale bar = 40 μ m. **B–D**) Quantitative comparison of staining for Myc and Ran in different tumors for staining for nuclear c-Myc and for cytoplasmic Ran (**C**), and staining for nuclear c-Myc and overall Ran (**D**). Fisher exact test (**B**, **C**) and χ^2 test (**D**) were used. **E–J**) Comparison of relative levels of Myc and Ran mRNA in lung cancer datasets available from the Gene Expression Omnibus (GEO) database. **E–I**) Relative mRNA levels of Myc and Ran was extracted from the GEO database and normalized by using R and Bioconductor as previously described (**8**). Datasets with patient number greater than or equal to 90 were included, and the correlations between relative mRNA levels of Myc and Ran in each dataset were compared by nonparametric bivariable correlation analysis (Spearman

rank test) using SPSS. In all the datasets tested in this study—GSE3141 ($n = 111$) (**E**), GSE4573 ($n = 130$) (**F**), GSE8894 ($n = 138$) (**G**), GSE13213 ($n = 117$) (**H**), and GSE14814 ($n = 90$) (**I**)—the relative mRNA levels of Myc and Ran were significantly positively correlated (Spearman rank test; $P < .05$). Note that the values for Myc and Ran in the GSE13213 dataset were negative because the microarray was performed using a two-channel method where the reference sample was RNA extracted from 20 lung cancer cell lines; the results indicated that most of the human specimens had a lower expression of Myc and Ran compared with the reference sample (20 lung cancer cell lines). **J**) The five datasets were combined into one by stratifying the mRNA levels of Myc and Ran into four groups equally using the three quartiles as cut-off points. In the combined lung cancer dataset ($n = 586$), the association between mRNA levels of Myc and Ran was tested by Spearman rank test. Statistically significantly more patients with a higher level of Myc mRNA also had a higher level of Ran mRNA (χ^2 test; $P < .001$). All statistical tests were two-sided.

and **C**, available online). The protein and mRNA expression levels of Ran and Myc were also analyzed in our lung cancer patient cohort and five independent lung cancer datasets available in the Gene Expression Omnibus database. As shown in **Figure 7**, increased staining of nuclear Myc protein was statistically significantly associated with nuclear (Spearman rank test, $P = .002$) (**Figure 7, A and B**), cytoplasmic ($P = .01$) (**Figure 7, A and C**), and overall ($P = .001$) (**Figure 7, A and D**) staining for Ran protein. Similarly, the level of Ran mRNA was statistically significantly correlated with that of Myc mRNA in all five independent lung cancer datasets analyzed ($P < .05$) (**Figure 7, E–I**). Importantly, when the mRNA levels were stratified into four equal groups and the five datasets were combined, a statistical significant association between Myc and Ran mRNA levels in lung cancer specimens was also observed ($P < .001$) (**Figure 7J**).

Association of Ran Expression and Patient Survival in Myc-Overexpressing Lung Cancers

Similar to the breast cancer patients, in lung cancer patients with a high immunohistochemical staining for nuclear Myc, a high level of staining for Ran protein was statistically significantly associated with a shorter survival time (Wilcoxon–Gehan test, $P = .048$; univariable Cox regression, $\chi^2 = 6.04$, RR = 2.8, 95% CI = 1.2 to 6.3,

$P = .01$) (**Figure 8A**). In contrast, no statistical significant association between Ran expression and survival was observed in patients with a low-level staining for nuclear Myc (Wilcoxon–Gehan test, $P = .20$) (**Figure 8B**). When analyzing the combined lung cancer datasets, a high level of Ran expression was statistically significantly associated with poorer survival (Wilcoxon–Gehan test, $P = .001$; univariable Cox regression, $\chi^2 = 11.0$, RR = 1.55, 95% CI = 1.12 to 2.00, $P = .01$) (**Supplementary Figure 5B**, available online). Moreover, in patients expressing a high level of Myc mRNA, a high level of Ran expression was also statistically significantly associated with a shorter survival time (Wilcoxon–Gehan test, $P = 0.001$; univariable Cox regression, $\chi^2 = 8.17$, RR = 1.99, 95% CI = 1.24 to 3.19, $P = .01$) (**Figure 8C**). Again, Ran mRNA expression was not statistically significantly associated with survival in patients with a low level expression of Myc (Wilcoxon–Gehan test, $P = .12$) (**Figure 8D**). Together, these results suggest that Ran expression may be an important determinant of Myc-mediated lung cancer progression.

Discussion

Previously, we have shown that Ran silencing results in more potent apoptotic response in cancer cells with activated PI3K/

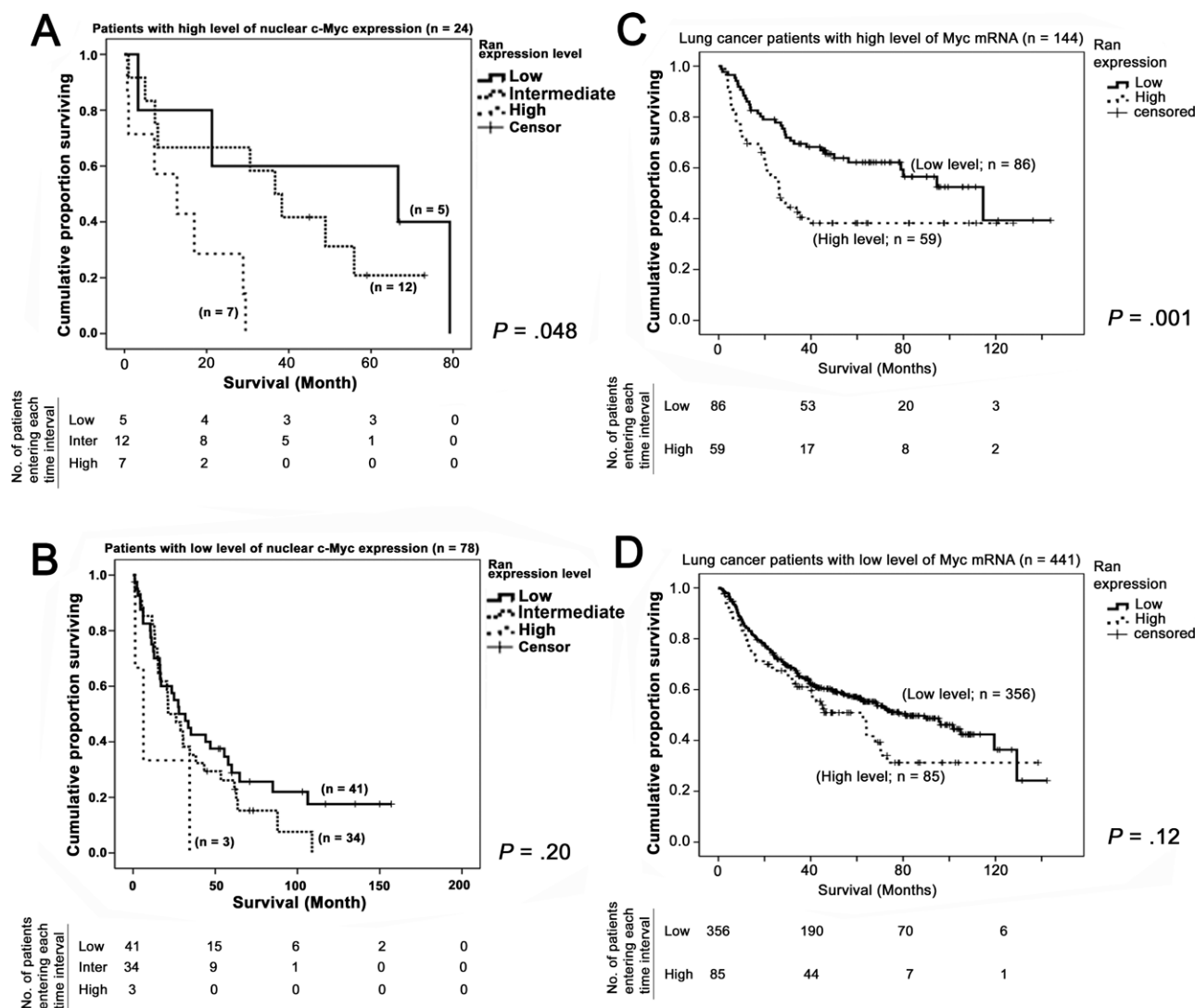


Figure 8. Association of expression of v-myc myelocytomatosis viral oncogene homolog (Myc) and Ras-related nuclear protein (Ran) alone and in combination in lung cancer patients. **A** and **B** Kaplan–Meier plots of the cumulative proportion of patients surviving with time in months of the Dublin lung cancer patient cohort are shown for tumors stained for low (**A**) and high (**B**) levels of nuclear Myc for high, intermediate, and low levels of overall (cytoplasmic and nuclear) Ran protein expression. **C** and **D** Kaplan–Meier plots of the cumulative proportion of patients surviving with time in months of the combined lung cancer dataset (n = 586) from Gene Expression Omnibus database for mRNA level of Ran in patients with low (**C**) and high (**D**) levels of Myc mRNA. Wilcoxon–Gehan test was used. All statistical tests were two-sided.

Akt/mTORC1 and Ras/MEK/ERK pathways. In this study, we investigated the role in myc-induced tumorigenesis in both breast and lung cancers, independent of instant apoptotic response, by using a less potent shRNA for Ran knockdown. In this study, we have demonstrated in immortalized breast epithelial MCF10A cells and breast cancer MDA MB 231 cells that both Ran and Myc in vitro are important in enhancing cellular properties, including colony growth in soft agar, cell adhesion, and cell invasion, associated with cancer aggressiveness. When the upstream region of Ran is examined, Myc binds to the distal, but not the proximal, E-box element and can transactivate the Ran promoter to upregulate Ran expression. The Myc–Ran promoter interaction has also been demonstrated in two other reports using a ChIP sequencing technique (35,36). We have further demonstrated that the in vitro observations are of clinical relevance by showing that Myc and Ran protein levels are

significantly positively correlated in 288 breast and 102 lung cancer patients and that their mRNA levels are also significantly positively correlated in 1454 breast and 586 lung cancer patients. In vitro, Ran knockdown suppresses the Myc-enhanced colony formation in soft agar in an immortalized breast epithelial cell line. Most important, we have shown that in both breast and lung cancer patients with a high level of Myc expression within the primary tumors, Ran overexpression is associated with increased death rates. These results provide clinical support to our findings in cultured cells that Ran may be an important downstream effector of Myc-mediated cancer progression.

In this study, we have also found that Ran is a direct transcriptional target of Myc and that a high level of Ran expression is important for the function of Myc overexpression in breast cancer cells in vitro and in Myc-overexpressing cancer patients. Myc is overexpressed in more than 40% of human cancers (37) and is,

therefore, a good target for cancer therapy. Our results suggest a novel approach for targeting Myc-overexpressing tumors in that silencing of Ran expression may inhibit Myc-driven cancer progression. Identification of small molecules that inhibit Ran-RCC1/Ran-RanGAP interactions or that downregulate Ran expression is underway, and these molecules could be tested in vitro and in vivo to validate our hypothesis.

In this study, we have shown that Ran expression is important in Myc-overexpressing breast and lung cancers. However, this study also had some limitations. The study was retrospective, and, although designed as intent-to-treat, the actual treatments of patients were heterogeneous. Small molecules that inhibit Ran expression or functions are yet to be identified. The investigation of these molecules on inhibiting cancer progression in human patients in a prospective setting is therefore highly warranted.

In our previous report (8) on breast and lung cancers, we showed that a high level of Ran expression is associated with a shorter survival time in patients, particularly those with K-Ras-activating mutations, with overexpression of c-Met and osteopontin, and with a PIK3CA activating gene signature, and in this study, we have shown this association in patients with Myc overexpression. These results also suggest that a high level of Ran expression is required for cancers driven by the common oncogenic mutations to express their aggressive behavior. Therefore, Ran may be an important therapeutic target for cancer patients harboring these oncogenic changes. In vitro experimental results from us and from others, obtained by silencing Ran, further support the clinical association. Cancer cells are more susceptible to Ran silencing than normal cells (6), whereas colon cancer cell lines harboring K-Ras-activating mutation are more susceptible to Ran silencing than the K-Ras wild-type isogenic counterparts (7,8). In conclusion, our data together suggest that Ran is a major downstream target of Myc-overexpressing cancer cells and that Ran may be a novel therapeutic target for cancer cells with oncogenic changes, including, but not limited to, Myc overexpression.

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